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The Bacterial Oxidation of Nicotine

I. NICOTINE OXIDATION BY CELL-FREE PREPARATIONS*

L. I. HOCHSTEIN† AND SYDNEY C. RITTENBERG

From the Department of Bacteriology, University of Southern California, Los Angeles, California

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Various pathways have been postulated for nicotine degradation involving an initial attack at either the pyridine or the pyrrolidine rings. The evidence for the suggested pathways has been provided mainly by the isolation of a variety of compounds from bacterial growth media (1, 2), tobacco seed infusions (3), fermented tobacco leaves (4), and the urine of animals that previously had been fed nicotine (5, 6). Unfortunately, because of the complexity of these systems it is not certain whether the isolated products are directly or indirectly derived from nicotine, nor is the sequence of their appearance clearly established. In an effort to avoid the difficulties inherent in the use of complex systems, studies of nicotine metabolism were attempted at the enzyme level employing crude and fractionated extracts derived from a bacterium.

EXPERIMENTAL

The organism (designated as strain P-34) employed is a gram-negative rod isolated from soil by enrichment culture techniques. It is capable of using nicotine as its sole source of carbon and energy but its growth is stimulated by yeast extract. It was grown in a medium having the following composition in grams per 100 ml.: 1.33 $K_2HPO_4 \cdot 3H_2O$, 0.4 KH_2PO_4, 0.1 $(NH_4)_2SO_4$, 0.1 yeast extract, 0.4 nicotine, and the following trace salts; 0.01 $MgSO_4 \cdot 7H_2O$, 0.002 $CaCl_2 \cdot 2H_2O$, 0.004 $MnSO_4 \cdot 4H_2O$, 0.0002 $FeSO_4 \cdot 7H_2O$. The trace salts were dissolved in 0.1 N HCl at 100 times the final medium concentration, autoclaved, and added aseptically to the medium in the required amounts.

For the preparation of large batches of cells, growth was carried out in 12-liter round bottom flasks containing 7 liters of medium. The inoculated medium was aerated with sterile air and incubated at room temperature. After the culture reached the maximum stationary phase (approximately 60 hours), the cells were harvested in a Sharples centrifuge. The unwashed cell paste was stored at −18° until needed. Yields were of the order of 6.5 gm. wet weight of cells per liter of medium.

Cell-free extracts were prepared according to the method of McIlwain (7) by grinding a mixture of 10 gm. of partially thawed cells and 25 gm. of levigated alumina in a cold mortar. After grinding for 10 to 14 minutes at room temperature, the cell-alumina mixture became "tacky." The resulting paste was extracted with 30 ml. of 0.01 M potassium phosphate buffer, pH 7, for 15 minutes at room temperature and for 45 minutes at 4°. The alumina, unbroken cells, and cell debris were removed by centrifugation at 18000 × *g* for 60 minutes at 4°. The resulting clear yellow supernatant fluid constituted the crude extract and contained, on the average, 22 mg. of protein per ml.

Ammonium sulfate fractionation of the crude extract was carried out at room temperature by the addition of the required amount of solid ammonium sulfate (8). After the addition of the ammonium sulfate, the precipitated protein was removed by centrifugation at 4° for 10 minutes at 18000 × *g*. The supernatant solution was decanted, the pellet drained by inversion and dissolved in 0.01 M potassium phosphate buffer, pH 7, to give a final volume of approximately one-fourth that of the starting crude extract. All enzymatic fractions were stored at −18° if not used immediately.

Protein was determined by trichloroacetic acid precipitation (9), crystalline egg albumin being used as the standard. The optical density at 540 mμ was determined in a Bausch and Lomb Spectronic-20.

Ultraviolet absorption spectra were determined with a Beckman model DU spectrophotometer. Reaction mixtures were prepared for spectrophotometry by adding them to 2 volumes of 0.1 N HCl, removing the precipitated protein by centrifugation, and, if required, diluting the clear supernatant solution with additional 0.1 N HCl.

Oxygen consumption and carbon dioxide production were determined by conventional manometric techniques (10).

Reaction mixtures were chromatographed on Whatman No. 1 paper by the ascending technique. The solvent was an 85:5:30 mixture of *n*-butanol, benzene, and 0.2 M sodium acetate buffer, pH 5.6 (11). The alkaloids were located by exposing the dried chromatograms to cyanogen bromide vapors for 1 hour followed by spraying with β-naphthylamine (a Koenig's reaction), by ultraviolet absorption under a Mineralite lamp, or by treating the paper with Dragendorff's reagent.[1]

RESULTS

Oxidation of Nicotine by Crude Extracts—Crude extracts prepared from several independently grown batches of cells usually oxidized nicotine at a slow but definite rate. The amount of oxygen consumed varied from extract to extract and in many cases no oxidation was observed. Furthermore, fresh extracts which oxidized nicotine lost this ability upon storage at −18°.

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† Present address, Department of Medical Microbiology, University of Southern California School of Medicine.

[1] Prepared by mixing 5 parts of solution A (0.85 gm. of bismuth subnitrate, 40 ml. of distilled water, and 10 ml. of glacial acetic acid) with 5 parts of solution B (8 gm. of potassium iodide in 20 ml. of distilled water), and adding 20 ml. of glacial acetic acid and 100 ml. of distilled water.

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TABLE I

Oxidation of nicotine by methylene blue-supplemented crude extracts

Experimental conditions: 1 μmole nicotine, 102 μmoles potassium phosphate buffer, pH 7, 1 μmole methylene blue where noted, 1 ml. crude extract; total volume 2.0 ml., gas phase air, 30°. The age of the extract represents days of storage, after preparation, at −18°.

Experiment No.	Age of extract	Methylene blue	Oxygen consumed after 60 min.
	days		*μl.*
1	0	−	48
		+	78
	4	−	2
		+	65
2	0	−	0
		+	71
	1	−	0
		+	67

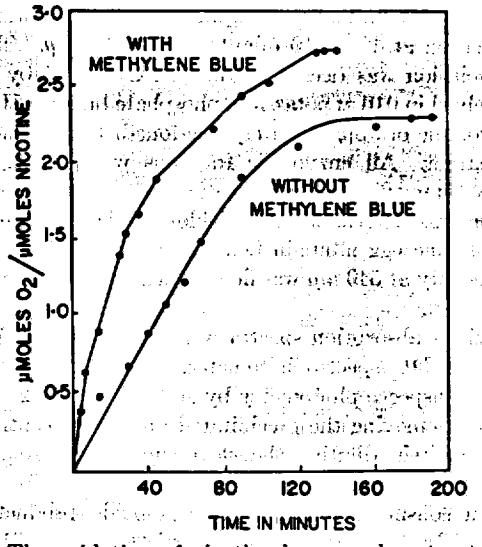

FIG. 1. The oxidation of nicotine by a crude extract. In the presence of methylene blue, the reaction mixture contained 5 μmoles of nicotine, 120 μmoles of potassium phosphate buffer, pH 7, and 1 ml. of crude extract. In the absence of methylene blue, the reaction mixture contained 1 μmole of nicotine, 88 μmoles of potassium phosphate buffer, pH 7, and 1 ml. of crude extract. Data corrected for oxygen consumption in the absence of substrate. Total volume, 2.0 ml., gas phase air, 30°.

TABLE II

Oxygen consumption and carbon dioxide formation during nicotine oxidation by methylene blue-supplemented crude extracts

Experimental conditions: 5 μmoles nicotine, 120 μmoles potassium phosphate buffer, pH 7, 1.25 μmoles methylene blue, 11 mg. (Experiment 1) and 17.3 mg. (Experiment 2) crude extract; total volume 2.0 ml., gas phase air, 30°.

Experiment No.	μMoles oxygen consumed		μMoles carbon dioxide produced	
	Total	Per μmole of nicotine	Total	Per μmole of nicotine
1	14.5	2.9	1.94	0.39
2	13.8	2.8	1.88	0.38

Oxidative activity in aged extracts was restored by the addition of methylene blue, brilliant cresyl blue, and 2,6-dichlorophenolindophenol, but not by a number of cofactors, by metallic ions,[2] or by cell debris.

In addition to restoring oxidative activity lost during storage, methylene blue rendered active those extracts which did not oxidize nicotine when freshly prepared, and stimulated the rate of oxygen consumption in those extracts which initially had oxidative activity (Table I). No oxidation of nicotine was observed in the absence of enzyme thus ruling out a photochemical oxidation of nicotine by methylene blue (12) under the conditions employed.

Methylene blue also affected the manner in which nicotine oxidation occurred. Whereas unsupplemented extracts oxidized nicotine at a constant rate, methylene blue-supplemented crude extracts oxidized nicotine in a series of steps of decreasing oxidative rate (Fig. 1). These rate changes occurred after the consumption of nearly 0.5 μmole of oxygen, or some integral multiple of 0.5, per μmole of nicotine until a total of approximately 3 μmoles of oxygen per μmole of nicotine was consumed. The data presented indicate rate changes after the consumption of 0.6, 1.6, 1.9, 2.5, and 2.8 μmoles of oxygen per μmole of nicotine. The position of the changes differed from extract to extract, and, in most experiments, the first change in rate occurred after the consumption of 1 μmole of oxygen per μmole of nicotine. Total oxygen consumption was usually 3 μmoles per μmole of nicotine, but occasionally somewhat smaller or larger values were observed. Assuming that 2 electrons were transferred per oxidative step, the typical crude extract catalyzed six oxidative reactions in nicotine degradation. No significant amounts of carbon dioxide were released up to this point of oxidation (Table II), suggesting that the fundamental carbon skeleton of nicotine might still be intact.

Chromatography of Reaction Mixtures—Chromatograms run on reaction mixtures stopped at the first rate change (after the consumption of approximately 0.5 μmole of oxygen per μmole of nicotine) showed no residual nicotine, but did show another compound with a lower R_F. After the consumption of approximately 1 μmole of oxygen per μmole of nicotine, neither nicotine nor the previous compound was detected, and a single new ultraviolet light-absorbing and Dragendorff reagent-positive spot was observed (Table III).[3] It would appear not only that products had accumulated at the points at which change of rate occurred, but also that essentially all of the initial substrate was consumed before oxidation of the subsequent substrate was initiated.

Changes in Ultraviolet Absorption Spectra—With the consumption of 0.5 μmole of oxygen per μmole of nicotine, a marked change in the ultraviolet absorption spectrum of the reaction mixture was observed. The absorption maximum at 260 mμ due to the pyridine moiety of nicotine disappeared, and in its place 2 absorption maxima appeared, a rather sharp and intense peak at 232 mμ, and a peak of lower extinction at 295 mμ. These peaks were absent after the consumption of 1 μmole of

[2] The following cofactors and metallic ions were tried individually and in combination with negative results: ATP, ADP, DPN, TPN, CoA, GSH, riboflavin, riboflavin-5-phosphate, cytochrome C, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $FeCl_2$, $ZnSO_4 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, and MoO_3.

[3] Although the observed R_F values were very close, the compound with an R_F of 0.12 gave a much redder color with Dragendorff's reagent than the compound with an R_F of 0.09.

TABLE III

Paper chromatography of reaction mixtures after consumption of 0.5 and 1 μmole of oxygen per μmole of nicotine

Oxygen consumed	R_F		
	CNBr	Ultraviolet absorption	Dragendorff's reagent
μmoles/μmole nicotine			
0	0.33	*	*
0.57	–†	0.12	0.12
1.05	–†	0.09	0.09
Nicotine control	0.32	0.32	0.32

* Not employed.
† No spot detected.

oxygen per μmole of nicotine. However, a new absorption maximum at 290 mμ was observed (Fig. 2). These results supported the chromatographic evidence for the temporary accumulation of intermediates, and showed that the first two of these substances could be easily distinguished from one another and from nicotine by means of their absorption in the ultraviolet.

Oxidation of Nicotine by Ammonium Sulfate Fractions—By fractionating the crude extract with ammonium sulfate in increments of 20 per cent of saturation with respect to ammonium sulfate, two preparations were obtained which oxidized nicotine when supplemented with methylene blue. Although fractionation did not lead to a large increase in specific activity, it did recover a major portion of the initial crude activity (Table IV). What is more important, fractionation with ammonium sulfate separated the material responsible for the initial oxidative step from the subsequent ones (Fig. 3).

The 20 to 40 fraction, when dissolved in buffer, gave an intensely yellow solution. It oxidized nicotine in the presence of methylene blue, and oxidation ceased after the consumption of 0.5 μmole of oxygen per μmole of nicotine (Fig. 3).

When nicotine and methylene blue were present in excess, the rate of nicotine oxidation was limited by the concentration of enzyme protein over the experimentally determined range of from 0.11 mg. to 1.1 mg. per ml. In the presence of an excess of nicotine and of enzyme, methylene blue limited the rate of oxidation up to a concentration of 2.5×10^{-4} M, in reasonably close agreement with that observed with crude extracts. In the presence of an excess of enzyme and of methylene blue, nicotine limited the rate of oxidation up to a concentration of approximately 5×10^{-3} M (Fig. 4).

After the oxidation of nicotine by the 20 to 40 fraction ceased, the ultraviolet absorption spectrum and the chromatographic behavior of the reaction mixture were identical to that observed at the 0.5 μmole oxygen rate change when nicotine was oxidized by a crude extract.

The 40 to 60 fraction was obtained as a light green pellet. Relatively concentrated solutions of this fraction were deep green in color when dissolved in 0.01 M potassium phosphate buffer, pH 7. This fraction oxidized nicotine only in the presence of methylene blue, with the consumption of 1 μmole of oxygen per μmole of nicotine (Fig. 3). At the end of the oxidation, the reaction mixture had the ultraviolet absorption spectrum and the chromatographic properties present at the 1 μmole oxygen rate change obtained with the crude extract. The 40 to 60 fraction apparently contained the enzymes responsible for both the first and second oxidative steps in nicotine degradation. The properties of this fraction will be more fully described in a subsequent paper.

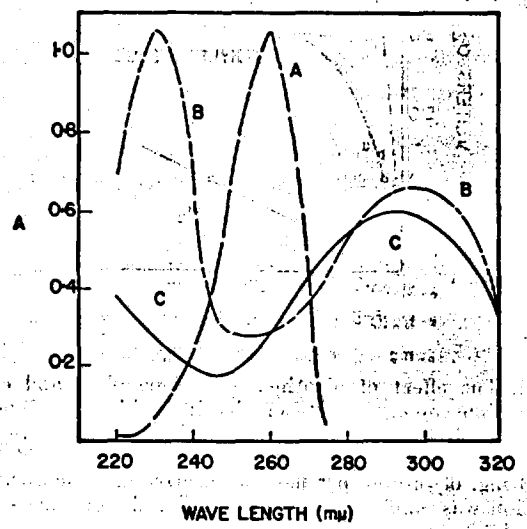

FIG. 2. The ultraviolet absorption spectra of reaction mixtures. *Curve A*, zero time control. *Curve B*, after the consumption of 0.53 μmole of oxygen per μmole of nicotine. *Curve C*, after the consumption of 1.04 μmoles of oxygen per μmole of nicotine.

TABLE IV

Fractionation of crude extracts with ammonium sulfate

Ammonium sulfate fraction	Total protein	Total units*	Specific activity	Recovery of units
% saturation	*mg.*		*units/mg.*	*%*
Crude	543	4724	9	
0–20	7	0	0	0
20–40	120	2712	23	57
40–60	222	1376	6	29
60–80	3	0	0	0

* A unit of enzyme activity is defined as 1 μl. oxygen consumed per 10 minutes during the maximum rate of oxygen consumption

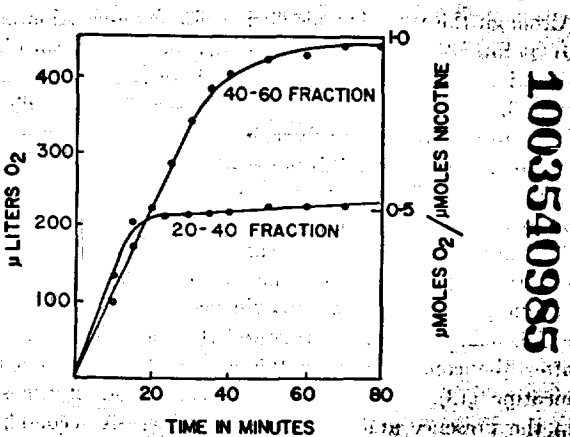

FIG. 3. The oxidation of nicotine by ammonium sulfate fractionated crude extracts. The reaction mixture contained 20 μmoles of nicotine, 58 μmoles of potassium phosphate buffer, pH 7, 1.25 μmoles of methylene blue, and the following ammonium sulfate fractions: 7.5 mg. 20 to 40 fraction and 18.5 mg. 40 to 60 fraction. No oxygen consumption was observed in the absence of nicotine, methylene blue, or the enzyme fractions.

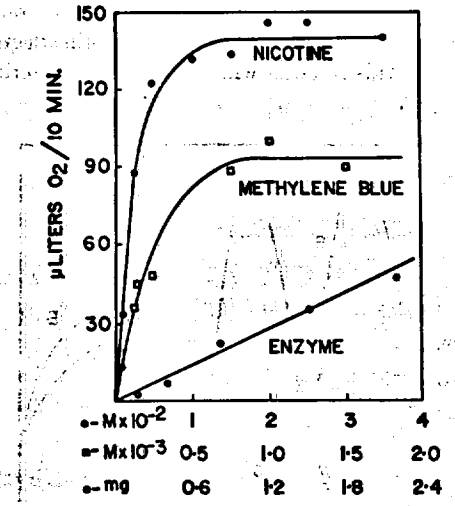

FIG. 4. The effect of nicotine, methylene blue, and enzyme concentrations upon the rate of nicotine oxidation. When not employed as the variable, the concentrations of nicotine, methylene blue, and the 20 to 40 fraction were 2×10^{-2} M, 1×10^{-3} M, and 28.4 mg. of protein per flask, respectively. When the 20 to 40 fraction was employed as the variable, the enzyme solution contained 2.2 mg. of protein per ml.

DISCUSSION

The observed changes in rate during oxidation of nicotine by crude extracts in the presence of methylene blue are puzzling. One could devise a model system exhibiting similar changes in rate in which a series of single step oxidations proceed simultaneously but at successively decreasing rates. In such a system, the rate of oxygen uptake would change with the disappearance of each member of the sequence in turn, and at the change, all succeeding members would be present in proportion to the difference in their rates of formation. Thus if

$$A \xrightarrow{3r} B \xrightarrow{2r} C \xrightarrow{1r} D \qquad (1)$$

at the indicated rates, r, the initial rate of oxygen uptake would be $6r$ and would change to $3r$ with the exhaustion of A. At this point, B, C, and D would be present in a ratio of 1:1:1. Although the rate changes during nicotine oxidation correspond to points of temporary accumulation of intermediates, the situation is quite different from the hypothetical example in that the data show that none of the serially accumulated products are metabolized until their successive precursors have been exhausted. That is, B is not oxidized until A is exhausted.

The mechanism responsible for this unique situation is not known. The possibility that each precursor competes with its product for an enzyme site and thus inhibits product oxidation was ruled out, at least in the case of the 0.5 μmole of oxygen product, by the following evidence. The 40 to 60 fraction, which oxidizes both nicotine and the product that accumulates after the consumption of 0.5 μmole of oxygen per μmole of nicotine (13), oxidized the latter compound at the same rate in the presence and absence of nicotine. A second possibility is that accumulation and changes of rate are a result of some cofactor deficiency, a cofactor shared in common by several of the enzymes involved in nicotine oxidation. This mechanism would demand that the cofactor exist as a ternary complex between enzyme, substrate, and cofactor, and that the enzyme-substrate complex have a very high affinity for the cofactor relative to the affinity of the succeeding enzyme-substrate complex.

The changes in the absorption spectrum during nicotine oxidation provide a clue as to the nature of the initial oxidative step. Nicotine, by virtue of its pyridine moiety, absorbs strongly at 260 mμ. It is known (14) that the introduction of a double bound in conjugation with the pyridine ring results in the appearance of a new absorption maximum at 234 mμ accompanied by a bathochromic shift of the 260 mμ absorption maximum. The addition of a second double bond in conjugation with the pyridine ring results in an additional bathochromic shift accompanied by a complete loss of the characteristic absorption maximum associated with the pyridine nucleus (14). One possible interpretation of our spectrophotometric data is that the initial reaction during the oxidation of nicotine produces an unsaturation of the pyrrolidine ring. However, the failure to observe a Koenig's reaction subsequent to the consumption of 0.5 μmole of oxygen per μmole of nicotine (Table III) implicates either the nitrogen or the α-carbons of the pyridine ring as the sites of attack (15). The two possible reactions in this case would be the addition of oxygen to the pyridine nitrogen to yield a pyridine-*N*-oxide derivative of nicotine, or the addition of oxygen to either α-carbon to yield a pyridone derivative of nicotine. The possibility that the product was an *N*-oxide seemed unlikely when it was found that pyridine-*N*-oxide itself, in 0.1 N HCl, has but a single absorption maximum located at 255 mμ (13). A pyridine-*N*-oxide derivative of nicotine would be expected to possess an analogous absorption spectrum in the ultraviolet. On the other hand, an authentic sample of 2-pyridone, in 0.1 N HCl, exhibited 2 absorption maxima, one at 227 and the other at 297; the latter absorption maximum had a lower extinction (13). Thus it seems probable that the first oxidative product is a pyridone and that the organism P-34 initiates the oxidation of nicotine in a manner similar to the pyridine pathway postulated by Frankenburg and Vaitekunas (3). In the following paper, the isolation and identification of this compound will be described.

SUMMARY

Crude cell-free extracts prepared from a soil bacterium capable of growing at the expense of nicotine as the sole source of carbon and nitrogen degraded nicotine with the consumption of 3 μmoles of oxygen per μmole of nicotine when supplemented with methylene blue. No carbon dioxide was formed up to this level of oxidation.

With crude extracts, nicotine oxidation proceeded through a series of sharp changes of rate occurring after the uptake of 0.5, or multiples of 0.5, μmole of oxygen per μmole of nicotine. Chromatographic evidence and ultraviolet absorption data indicated that each point of change of rate coincided with the temporary accumulation of intermediates in the oxidation sequence, and that none of the serially accumulated intermediates are oxidized until their precursors are exhausted. After the consumption of 0.5 μmole of oxygen per μmole of nicotine, with the use of either a crude extract or an ammonium sulfate fractionated enzyme, the absorption maximum at 260 mμ resulting from nicotine disappeared, and a compound having absorption peaks at 232 and 295 mμ appeared. With the additional uptake of 0.5 μmole of oxygen per μmole of nicotine, these peaks disappeared and a new absorption maximum at 290 mμ was observed.

The nature of the absorption spectrum of the first oxidative product and its failure to give a Koenig's reaction suggests a primary attack at the pyridine moiety of nicotine to yield a pyridone substituted at an α-carbon of the pyridine ring.

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